Characterization of engineered preQ1 riboswitches for inducible gene regulation in mycobacteria

Erik R. Van Vlack[a], Shana Topp[b] *, Jessica C. Seeliger[c] #

Department of Chemistry, Stony Brook University, Stony Brook, NY, USA[a]; Department of Chemistry, University of California, Berkeley, CA, USA[b]; Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY, USA[c]

Supplemental Information

Supplemental Methods

GFP fluorescence kinetic assay. Starter cultures of Msm were grown overnight to OD600 1 and then subcultured to OD600 0.02 in 5 ml of 7H9 medium containing preQ1. Cultures were incubated at 37 °C and 250 rpm. At each time point, 200 µl of each culture was removed and aliquoted into black clear-bottom 96-well plates. Fluorescence was recorded with an F5 Filtermax plate reader using 485 nm excitation and 535 nm emission filters. The OD595 was measured in parallel and used to normalize GFP fluorescence values. After 18 hours, the cells were spun down at 4000 rpm and resuspended at OD600 0.2 in fresh 7H9. This was repeated three times to remove preQ1. The resuspended cultures were allowed to incubate for three more doubling times, with fluorescence measured every 3 hours as above.

Derivation of steady-state equations for a two-state model with one or two binding sites. We assume that the equilibrium between conformational states of the riboswitch dominates the observed response and that the rates of transcription, translation, and protein and mRNA degradation can therefore be ignored. In the absence of ligand, a given mRNA transcript interconverts between the A and B conformational states according to equilibrium constant K_{int}, as shown in **Scheme 1** (Figure 2). It is assumed that transcripts in the B state cannot be translated. Only B binds to the ligand L with the dissociation constant K_d to form the complex BL, which, like the B state, is not competent for translation. From these equilibria we have the following relationships:

$$K_{int} = \frac{[B]}{[A]} \qquad \textit{Eq. S1}$$

$$K_d = \frac{[B][L]}{[BL]} \qquad \textit{Eq. S2}$$

which can be rewritten as

$$[B] = [A]K_{int} \qquad \textit{Eq. S3}$$

$$[BL] = \frac{[B][L]}{K_d} = \frac{[L]}{K_d}[A]K_{int} \qquad \textit{Eq. S4}$$

The concentration of total transcripts R is defined as

$$[R] = [A] + [B] + [BL] \qquad \textit{Eq. S5}$$

Under the assumptions of our model, the relative protein concentration $[P]_{rel}$ can be described as proportional to the ratio of transcripts in the A state to the total transcripts R:

$$[P]_{rel} \propto \frac{[A]}{[R]} = \frac{[A]}{[A]+[B]+[BL]} \qquad \textit{Eq. S6}$$

which can be simplified using Eq. S3 and S4 to yield:

$$[P]_{rel} = \frac{1}{1+k_{int}(1+\frac{[L]}{k_d})}$$ **Equation 1**

Finally, the terms *a* and *b* are introduced as a scaling factor and baseline respectively, to scale the relative protein concentration to the experimental observable. The relationship between the observed protein concentration, as represented by the normalized fluorescence F_{norm}, and the applied ligand concentration (which is assumed equal to the intracellular ligand concentration) is then:

$$F_{norm} = b + \frac{a}{1+K_{int}(1+\frac{[L]}{K_d})}$$ *Eq. S7*

The two-site model for riboswitch function is similarly derived from **Scheme 2** (Figure 2), wherein the thermodynamic constant K_{int} controls conversion between the A and the B states in the absence of ligand, but two dissociation constants, K_{d1} and K_{d2}, describe ligand binding to each of two different independent sites on the riboswitch. Then we have:

$$K_{int} = \frac{[B]}{[A]}$$

$$K_{d1} = \frac{[B][L]}{[BL]}$$ *Eq. S8*

$$K_{d2} = \frac{[B][L]}{[LB]}$$ *Eq. S9*

And therefore:

$$[B] = [A]K_{int}$$

$$[BL] = \frac{[B][L]}{K_{d1}} = \frac{[L]}{K_{d1}}[A]K_{int}$$ *Eq. S10*

$$[LB] = \frac{[B][L]}{K_{d2}} = \frac{[L]}{K_{d2}}[A]K_{int}$$ *Eq. S11*

Because the two binding equilibria are independent, $[P]_{rel}$ is then:

$$[P]_{rel} = \frac{1}{1+K_{int}(1+\frac{[L]}{K_{d1}})} + \frac{1}{1+K_{int}(1+\frac{[L]}{K_{d2}})}$$ **Equation 2**

which in terms of the observed fluorescence F_{norm} becomes

$$F_{norm} = b + \frac{a}{1+K_{int}(1+\frac{[L]}{K_{d1}})} + \frac{c}{1+K_{int}(1+\frac{[L]}{K_{d2}})}$$ *Eq. S12*

Table S1. Plasmid constructs and primers used in this study.

Restriction sites used for cloning: **BamHI**, **XbaI**, ***MscI***, HindIII, *ClaI*, *NdeI*

Name	Plasmid ID	Template	Vector	Method	Forward primer	Reverse primer 1	Reverse primer 2	Reverse primer 3
Lrh	pEV07	pMV261	pMWS114	NPP[†]	gatctttaaa**tctga**ggtgacca caacgacgcg	tcgtcgtggaagcgcgcggggcg gttcccaacgttattcttagcactcg cctatgccgag	ttgtggctttgccagtaataacgac gatcaaaggaaataagtatcgtc gtggaagcgcgc	gatccgcaattgtct***tggcca***tattgtt tctcctttgtggctttgccagtaataac gacg
Tte	pEV06	pMV261	pMWS114	NPP	gatctttaaa**tctga**ggtgacca caacgacgcg	tttgttaactggggttactgcgacc caggtgacaacgttattcttagca ctcgcctatgc	ccgcaattgtct***tggcca***tcttgtt gcctccttgttttgttaactggggtt actgcgacc	
Fpr	pEV37	pMV261	pMWS114	NPP	gatctttaaa**tctga**ggtgacca caacgacgcg	ccggggagcctaaaatcctaagtt gctccaacgttattcttagcactcg cctatgccgag	atgggtttggcccggcatggttcga gacacgccggggagcctaaaat cctaagttgctcc	tccgcaattgtct***tggcca***taacaat actccggcggatgaattttgacccac agggaccg
T1	pEV15	pEV06		SDM[††]	cctgggtcgaaacccttcagtaa ccccagttaacaaaacaagga ggcaac	ggggttactgaagggtttcgaccc aggtgacaacgttattcttagcact c		
T2	pEV14	pEV06		SDM	cctgggtcgaaaccttccagtaa ccccagttaacaaaacaagga ggcaac	ggggttactggaaggtttcgacc caggtgacaacgttattcttagca ctc		
T3	pEV13	pEV06		SDM	cctgggtcgaaactctccagtaa ccccagttaacaaaacaagga ggcaac	ggggttactggagagtttcgaccc aggtgacaacgttattcttagcact c		
T4	pEV12	pEV06		SDM	cctgggtcgaaaccctccagta accccagttaacaaaacaagg aggcaac	ggggttactggagggtttcgaccc aggtgacaacgttattcttagcact c		
T5	pEV25	pEV06		SDM	cctgggtcgaaacgtctccagta accccagttaacaaaacaagg aggcaac	ggggttactggagacgtttcgacc caggtgacaacgttattcttagcac tc		
T6	pEV24	pEV06		SDM	cctgggtcgaaacgcttccagta accccagttaacaaaacaagg aggcaac	ggggttactggaagcgtttcgacc caggtgacaacgttattcttagcac tc		
T7	pEV23	pEV06		SDM	cctgggtcgaaacctgtctccag taaccccagttaacaaaacaag	ggggttactggagacaggtttcga cccaggtgacaacgttattcttagc		

					gaggcaac	actc		
T8	pEV16	pEV06		SDM	cctgggtcgaaacgcctccagtaaccccagttaacaaaacaaggaggcaac	ggggttactggaggcgtttcgaccaggtgacaacgttattcttagcactc		
T9	pEV17	pEV06		SDM	cctgggtcgaaacctgcctccagtaaccccagttaacaaaacaaggaggcaac	ggggttactggaggcaggtttcgacccaggtgacaacgttattcttagcactc		
T10	pEV18	pEV06		SDM	cctgggtcgaaacacttgcctccagtaaccccagttaacaaaacaaggaggcaac	ggggttactggaggcaagtgtttcgacccaggtgacaacgttattcttagcactc		
T11	pEV19	pEV06		SDM	cctgggtcgaaacacagttgcctccagtaaccccagttaacaaaacaaggaggcaac	ggggttactggaggcaactgtgtttcgacccaggtgacaacgttattcttagcactc		
T1*	pEV27	pEV06		SDM	cctgggtcgaaacaagacagtaaccccagttaacaaaacaaggaggcaac	ggggttactgcuccgtttcgacccaggtgacaacgttattcttagcactc		
T0	pEV26	pEV06		SDM	aaggaggcaacctcatggccaagacaattgcggatccagctgcag	cttggccatgaggttgcctccttgtttttgttaactggggttactgcgacc		
L1	pEV08	pEV07	pMWS114	NPP	gatctttaaa**tctga**ggtgaccacaacgacgcg	tcgtcgtggaagcgcgcggggcggttcccaacgttattcttagcactcgcctatgccgag	ttgtggctttgccagtaataacgacgatcaaaggagataagtatcgtcgtggaagcgcgc	gatccgcaattgtct***tggcca***tattgtttctcctttgtggctttgccagtaataacgacg
L2	pEV09	pEV07	pMWS114	NPP	gatctttaaa**tctga**ggtgaccacaacgacgcg	tcgtcgtggaagcgcgcggggcggttcccaacgttattcttagcactcgcctatgccgag	tggctttgccagtaataacgacgatcaaaggaaaattataagtatcgtcgtggaagcgcg	gatccgcaattgtct***tggcca***tattgtttctcctttgtggctttgccagtaataacgacg
L3	pEV10	pEV07	pMWS114	NPP	gatctttaaa**tctga**ggtgaccacaacgacgcg	tcgtcgtggaagcgcgcggggcggttcccaacgttattcttagcactcgcctatgccgag	tttgccagtaataacgacgatcaaaggaaaaacttaaataagtatcgtcgtggaagcgcg	gatccgcaattgtct***tggcca***tattgtttctcctttgtggctttgccagtaataacgacg
L4	pEV11	pEV07		SDM	actggccgcaaagcggccacaaaggagaaacaatatggccaagac	gtggccgctttgcggccagtaataacgacgatcaaaggaaataagtatcgtcgtggaagc		
L5	pEV28	pEV07		SDM	actgggccgcaaagcggtccacaaaggagaaacaatatggccaagac	gtggaccgctttgcggcccagtaataacgacgatcaaaggaaataagtatcgtcgtggaag		
L6	pEV29	pEV07		SDM	actggaccgcaaagcggtcc	gtggaccgctttgcggtccagta		

					acaaaggagaaacaatatggccaagac	ataacgacgatcaaaggaaataagtatcgtcgtggaag		
L7	pEV30	pEV07		SDM	actggccgcgaaacgcggccacaaaggagaaacaatatggccaagac	gtggccgcgtttcgcggccagtaataacgacgatcaaaggaaataagtatcgtcgtggaag		
L8	pEV31	pEV07		SDM	actggccgccaaaggcggccacaaaggagaaacaatatggccaagac	gtggccgcctttggcggccagtaataacgacgatcaaaggaaataagtatcgtcgtggaag		
B1	pEV38	pST194		SDM	ggagaggttaatactccctagctacaccctctataaaaaactaaggaggt	gtagctagggagtattaacctctccccgcatatcgcacgg		
B2	pEV39	pST194		SDM	ggagaggttaatattccctagctacaccctctataaaaaactaaggaggt	gtagctagggaatattaacctctccccgcatatcgcacgg		
TetGFP	Tet-egfp	pTet-GW***	pMWS114	TL†††	catgcttaattaagaaggagatatacatatggccaagacaattgcg	gcttgatatcttacttgtacagctcgtccatgc		
GFPI	pEV40	pMWS114	pMV306**	TL	subcloned via XbaI, HindIII			
TteI	pEV41	pEV06	pMV306	TL	subcloned via XbaI, HindIII			
T3I	pEV42	pEV13	pMV306	TL	subcloned via XbaI, HindIII			
T4I	pEV43	pEV12	pMV306	TL	subcloned via XbaI, HindIII			
pRibo LprG-FLAG	pEV46	pMV261 LprG-FLAG	pEV43	SDM, then NPP	gcaaccata***tggcca***agacaattgcggat	ccatatggttgcctccttgttttgttaactggg	aaacaaggaggcaaccatatgcagacgcgcccac	cgacatcgataagctttattacttgtcgtcgtcgtccttgtag
LprG-FLAG	pEV47	pMV261 LprG-FLAG	pEV40	NPP	aaacaaggaggcaac*catatg*cagacgcgcccac	cgacatcgataagctttattacttgtcgtcgtcgtccttgtag		
pRibo-KatG-S	pEV48	*Msm* gDNA	pMV261	NPP	gacatcgataagcttctattcgggttgacgtagatgagg	ccagtaaccccagttaacaaaacaaggaggcaacaagttgcctgaggatcgcccg	ctaagaataagcttgtcacctgggtcgaaaccctccagtaaccccagttaacaaaacaag	

mCherry ****	pEV49	pCherry3 ****	pMV306	TL	atctttaaat**ctga**gattagctaagcagaaggc	ctacgtcgac*atcgat*cgataaaataaaaaagg		

* Reported in Seeliger *et al*.
** Addgene Plasmid #26155
*** Gift from Christopher Sassetti
**** mCherry From Carroll *et al.* (obtained from AddGene, Cat# 24659)
† NPP = nested primer PCR
†† SDM = site-directed mutagenesis
††† TL = traditional ligation

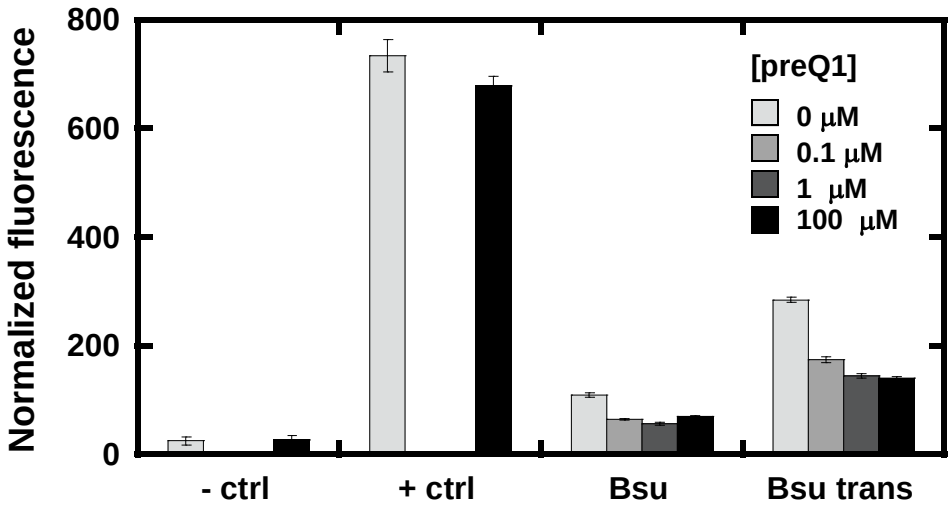

Figure S1. Significant riboswitch response to preQ1 occurs at [preQ1] below 100 μM preQ1. *Msm* expressing GFP under the control of the Bsu or Bsu Trans ribowitches were treated with 0, 1, 10, 100 and 1000 μM preQ1 for 6 h. GFP fluorescence was normalized to OD_{600}. Data are reported as the average of three technical replicates ± SEM. (Note: The scale of the normalized fluorescence values differ from other measurements reported in this study because these data were taken on a different instrument.)

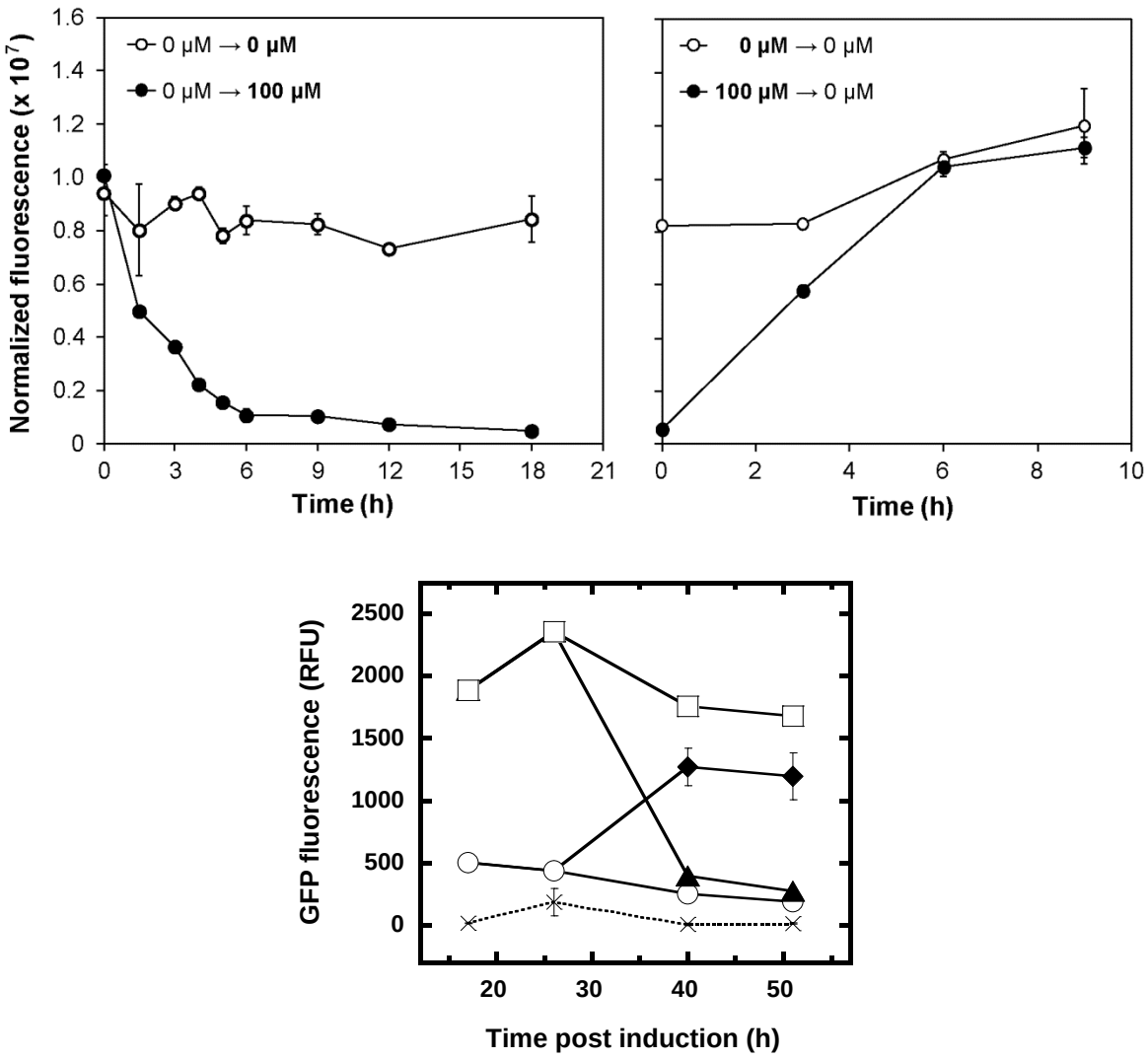

Figure S2. PreQ1-dependent gene repression is inducible and reversible in Msm and Mma. (TOP, *left*) GFP fluorescence in Msm whole cells expressing GFP regulated by the T1 riboswitch in response to preQ1 at 100 µM. Response was initiated by inoculation into preQ1-containing or preQ1-free medium at t = 0. (TOP, *right*) After overnight growth in preQ1-containing or -free medium, cells were pelleted and resuspended in preQ1-free medium at time = 0. Msm doubles approximately every 3h. (BOTTOM) GFP fluorescence in Mma whole cells expressing GFP regulated by the *Bsu trans* riboswitch in response to preQ1 at 100 µM. Response was initiated by inoculation into preQ1-containing (circles) or preQ1-free medium (squares) at t = 0. After 17 h (~3 doubling times), cells were pelleted by centrifugation and resuspended in fresh medium either maintaining the same preQ1 concentrations (open symbols) or switching to preQ1-containing (triangles) or preQ1-free (diamonds) medium. All cultures were incubated for a further 34 h (>5 doubling times). Mma transformed with empty vector (crosses) served as a negative control. Fluorescence was normalized to OD595 at each time point. Data are presented as mean ± SEM of 3 biological replicates.

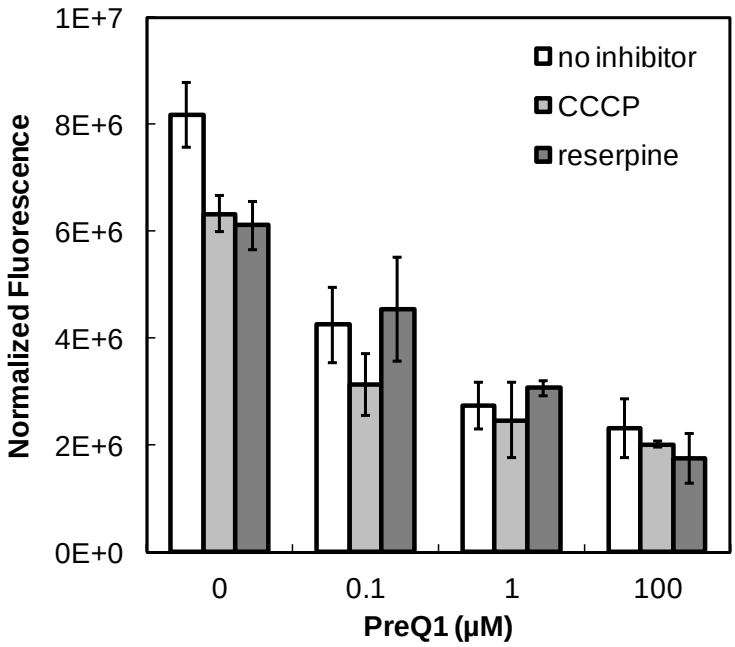

Figure S3. Inhibition of efflux does not affect preQ1-dependent gene regulation. GFP fluorescence normalized to OD_{595} in *Msm* whole cells expressing GFP controlled by the Bsu riboswitch in response to 0, 0.1, 1 or 100 μM preQ1 after 6 hours of treatment with 12 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or 21 μM reserpine (concentrations approximately 10X below the MIC as suggested by Jin *et al.*). Data are presented as mean ± SEM of three biological replicates.
.

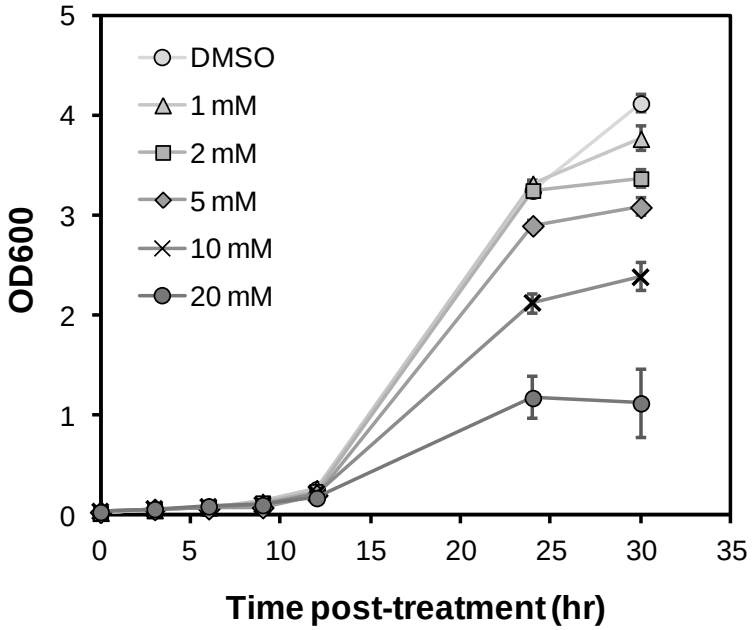

Figure S4. PreQ1 does not significantly affect *Msm* growth up to several millimolar. Wild-type Msm was cultured in 7H9 liquid medium supplemented with the specified concentrations of preQ1 or DMSO vehicle and growth was monitored via OD_{595}.

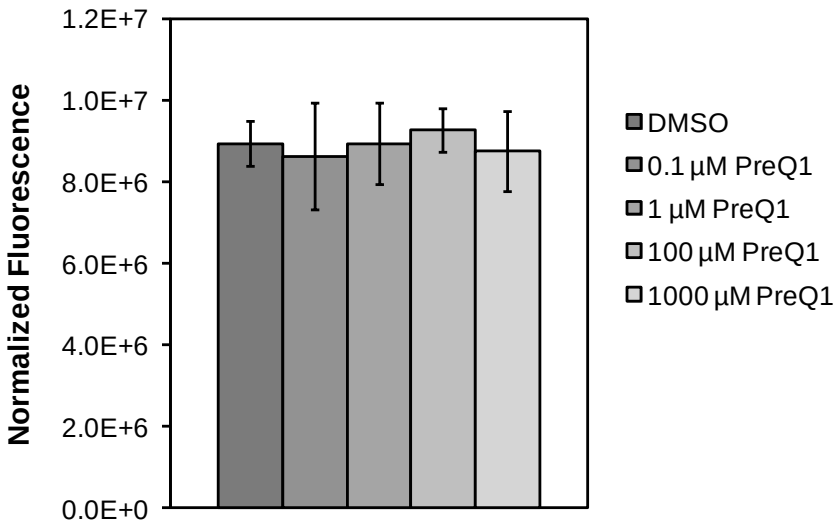

Figure S5. PreQ1 does not affect riboswitch-independent gene expression. GFP fluorescence normalized to OD_{595} in *Msm* whole cells expressing GFP under the control of the constitutive mycobacterial promoter P_{hsp60} in response to 0.1, 1, 100 or 1000 µM preQ1 after 6 hours of treatment. Final DMSO concentration was 1% in all samples. Data are presented as mean ± SEM of two biological replicates.

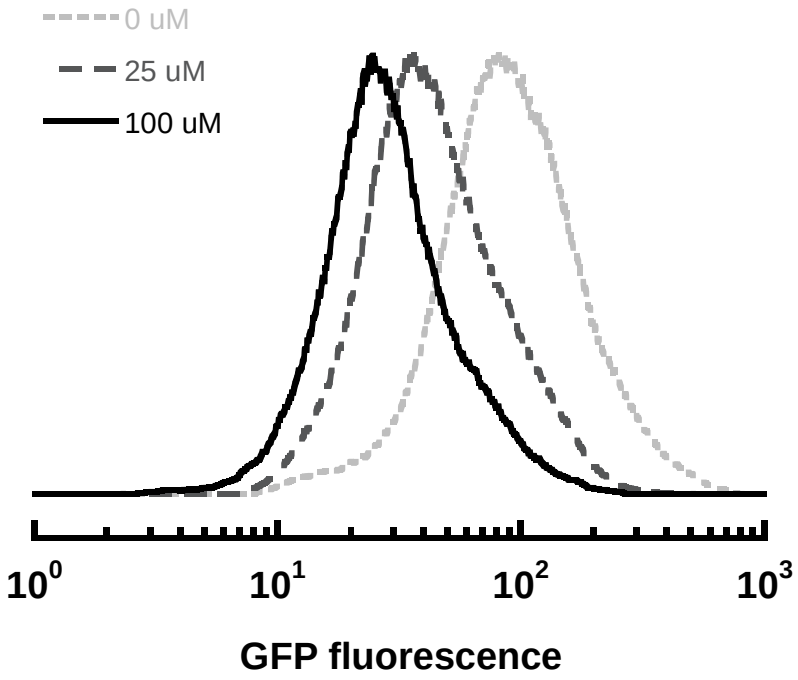

Figure S6. Riboswitch response to preQ1 is titratable. GFP fluorescence in Msm whole cells expressing GFP regulated by the *Bsu trans* riboswitch in response to preQ1 at 0, 25, and 100 µM. Flow cytometry was performed as in Seeliger et al. 2012.

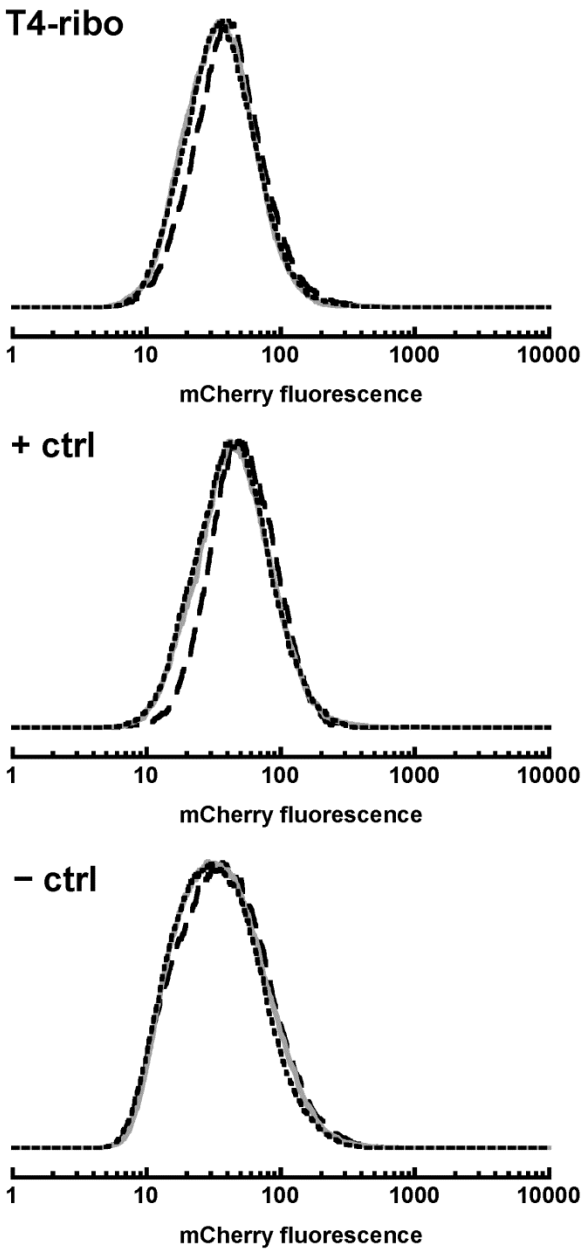

Figure S7. PreQ1 treatment does not affect bacterial load in *Msm*-infected macrophages. J774.1 macrophage-like cells were infected with *Msm* expressing mCherry constitutively and GFP constitutively (+ ctrl), under T4 riboswitch control (T4-ribo) or not at all (– ctrl). Treatment of infected cells with 0 (black dashed line), 0.1 (gray solid line), or 1 (black dotted line) mM preQ1 for 12 hours did not significantly affect the bacterial load, as assessed by the mCherry fluorescence.

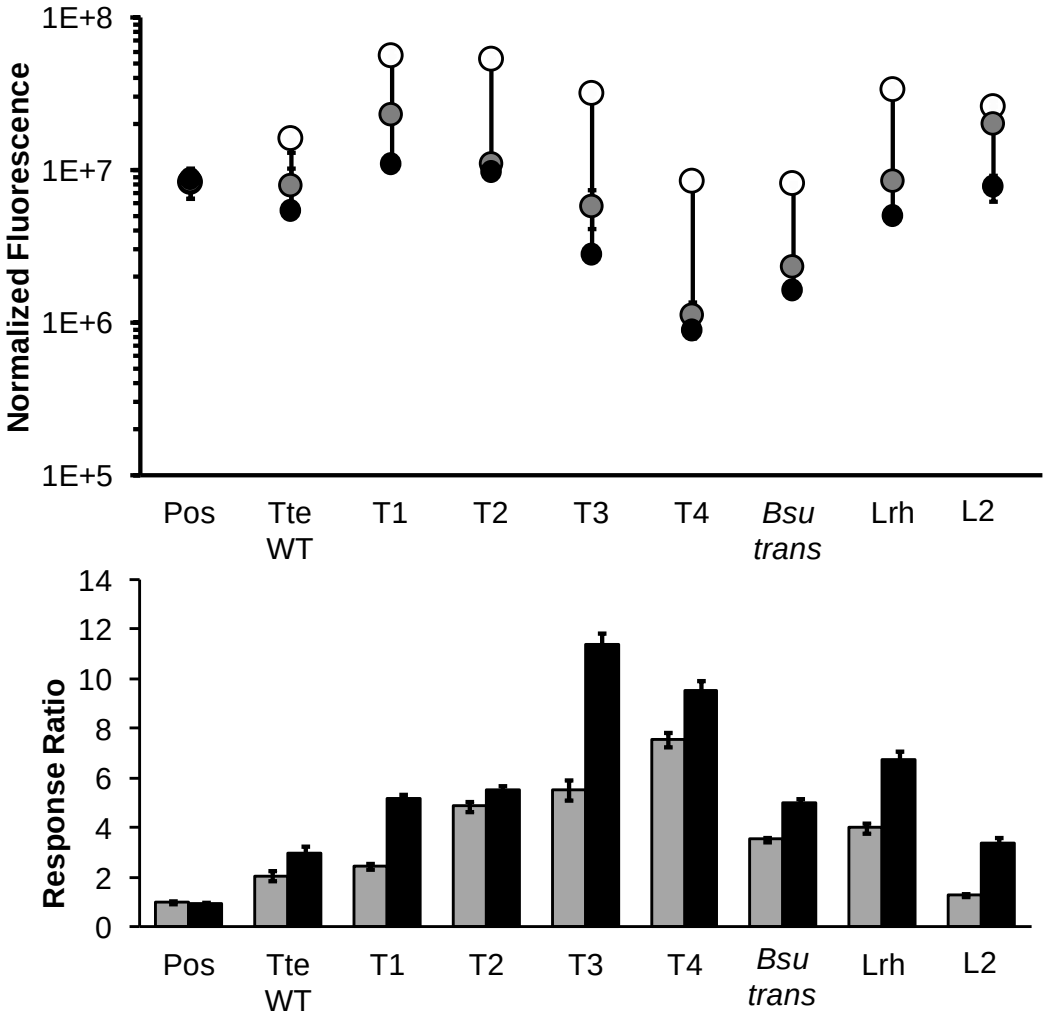

Figure S8. Response ratios are higher at 1 mM than at 100 μM preQ1. (*top*) GFP fluorescence normalized to OD_{595} in *Msm* whole cells after 6 hours in response to 0 μM (open circles), 100 μM (gray circles), or 1 mM (black circles) preQ1. (*bottom*) Response ratios at 100 μM (gray bars), or 1 mM (black bars) preQ1 calculated from the fluorescence data. Data at 1 mM preQ1 are from Figure 4. All data are presented as mean ± SEM of at least three biological replicates.

References

Chen X, Ellington AD. 2009. Design principles for ligand-sensing, conformation switching ribozymes. PLoS Comput Biol **5:**e1000620.

Jin J, Zhang JY, Guo N, Sheng H, Li L, Liang JC, Wang XL, Li Y, Liu MY, Wu XP, Yu L. 2010. Farnesol, a potential efflux pump inhibitor in Mycobacterium smegmatis. Molecules **15:**7750-7762.

Seeliger JC, Topp S, Sogi KM, Previti ML, Gallivan JP, Bertozzi CR. 2012. A riboswitch-based inducible gene expression system for mycobacteria. PLoS ONE **7:**e29266.

Stover CK, de la Cruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, Bansal GP, Young JF, Lee MH, Hatfull GF, et al. 1991. New use of BCG for recombinant vaccines. Nature **351:**456-460.

Carroll P, Schreuder LJ, Muwanguzi-Karugaba J, Wiles S, Robertson BD, Ripoll J, Ward TH, Bancroft GJ, Schaible UE, Parish T. 2010. Sensitive detection of gene expression in mycobacteria under replicating and non-replicating conditions using optimized far-red reporters. PLoS ONE **5:**e9823.